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Is Expressed in the Organizer and Regulates Nodal and Activin Signaling

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The latent TGF-β binding proteins (LTBP) are believed to control the availability of TGF-β in the extracellular milieu. To gain insight into the potential roles of LTBP in early development, we isolated the *Xenopus* LTBP-1 (xLTBP-1) cDNA. The cDNA encodes a protein similar to the mammalian LTBP-1 in both size and domain structure. In addition, we found a novel longer splice isoform of xLTBP. The RNAs for both forms of xLTBP displayed temporal regulation and the shorter transcript is expressed maternally. Both transcripts also display spatial regulation and are found in the dorsal mesoderm of the organizer. In animal cap experiments, LTBP-1 potentiates the activity of activin and nodal. The activity of LTBP-1 did not appear to require covalent association with activin as the addition of medium containing activin and LTBP-1 to animal caps enhanced the activin effect. These results indicate that LTBP-1 may be part of the regulatory system that establishes the threshold of morphogen activity for activins and nodals in the dorsal side of the embryo during gastrulation.

Key Words: **LTBP; activin; nodal; Xenopus; mesoderm; induction; morphogen; organizer.**

INTRODUCTION

Signaling by members of the TGF-β superfamily mediates key roles in embryonic development. In vertebrate embryos, multiple TGF-β-related ligands (32 in the human genome) are involved in a variety of activities, including the establishment of the germ layers, patterning events within germ layers, cell fate determination, axis determination, and cell movements. In recent years, the overall intracellular molecular signaling cascade initiated by these ligands has been elucidated (Moustakas *et al.*, 2001; Wrana, 2002). This includes binding to plasma membrane serine, threonine kinases, receptor cross phosphorylation, interaction of Smad proteins with the cytoplasmic domain of the receptors, phosphorylation of signaling Smads followed by dimerization with Smad4, and migration of the complex into the nucleus. Subsequent association of the Smad complex with a variety of transcription factors modifies gene expression both positively and negatively (Massague and Chen, 2000; Massague and Wotton, 2000).

Whereas the intracellular aspects of TGF-β signaling have received much attention, the mechanisms regulating ligand presentation to the receptor are less well characterized. It is believed that upon secretion, the TGF-β family members are bound to extracellular matrix proteins from which, by an as yet unknown mechanism, they are subsequently released according to need and presented to the receptors. Superimposed upon ligand presentation to its receptor after release is the action of secreted positive and negative regulators of both the growth factors and their receptors. For example, molecules such as noggin, chordin, follistatin, and cerberus are secreted by cells of the dorsal mesoderm bind to and directly inhibit BMPs, activins, and nodals. Also, the coreceptor one-eye pinhead (oep) is a positive regulator of nodal activity (Gritsman *et al.*, 1999; Shen and Schier, 2000).

Another group of TGF-β binding proteins that modulate growth factor availability is the latent TGF-β binding proteins (LTBP) that covalently bind to the TGF-β propeptide (Koli *et al.*, 2001; Saharinen *et al.*, 1999). TGF-β superfamily members are all synthesized as dimeric propro-

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teins in which the pro-domain dimer is cleaved intracellularly from the mature cytokine. TGF-βs 1, 2, and 3 are unusual as their proproteins remain associated with the mature cytokine by noncovalent interactions after secretion. In order for TGF-β to bind to its receptor, the cytokine must dissociate from the propeptide. The LTBPs are disulfide linked to a cysteine in the TGF-β propeptide. The LTBPs (1, 2, 3, and 4) are chordate inventions and belong to the extracellular matrix protein superfamily that includes the fibrillins and the LTBPs. Based on *in vitro* experiments, the LTBPs appear to facilitate TGF-β secretion (Miyazono *et al.*, 1991) and activation (Flaumenhaft *et al.*, 1989) as well as having a structural function in the ECM (Dallas *et al.*, 1995). A null mutation for murine LTBP-3 results in cranial facial and long bone defects, but embryonic development is normal and the animals are fertile (Dabovic *et al.*, 2002). These phenotypes are consistent with a defect in TGF-β presentation and support the proposed role for LTBP-1, -3, and -4 in modulating TGF-β availability. LTBP-2$^{-/-}$ mice die during embryogenesis (E3.5–E6.5), demonstrating an early requirement for the protein (Shipley *et al.*, 2000). Although LTBP-2 does not bind TGF-β (Saharinen and Keski-Oja, 2000), the early lethality of the mutant mice suggests a critical role for LTBP-2 in development, perhaps by binding to a signaling molecule by noncovalent interactions. Loss-of-function mutations for LTBP-1 and -4 have not been reported. Thus, despite biochemical progress, the function, order of interactions in the extracellular space, interactions with additional TGF-β superfamily members, and the biological integration of these functions of the LTBPs are poorly understood.

In this study, we report first the isolation of two isoforms of xLTBP-1 from *Xenopus*: a form equivalent to the molecule described in other species and a novel splice variant encoding a longer form. Both isoforms of xLTBP-1 are under zygotic control, and expression is observed during early gastrulation through the tadpole stage. In addition, the shorter transcript is expressed maternally. Second, both xLTBP-1 transcripts are expressed in dorsal mesoderm cells of the organizer, but the long form is specific for the dorsal marginal zone (DMZ). Third, xLTBP-1 synergizes with both activin and nodal in mesoderm induction. Fourth, there is selective interaction of LTBP-1 with activin and Xnr1. Fifth, secreted LTBP-1 protein can synergize with secreted activin, demonstrating for the first time that protein–protein interaction between LTBP-1 and TGF-β ligands is not restricted to the intracellular secretion pathways. These results highlight a role for LTBP-1 in modulating the activity of activin and nodal in the extracellular space during embryonic development.

MATERIALS AND METHODS

Cloning and RT-PCR

A Xenopus cDNA library made from XTC cells (Pudney *et al.*, 1973) was screened by using a probe derived from full-length human LTBP-1. Positive clones were sequenced and those containing DNA sequence representing the Xenopus homologue of LTBP-1 were identified. Specific primers were designed to assemble an open reading frame in the Xenopus expression vector CS2++ (Rupp and Weintraub, 1991; Turner and Weintraub, 1994). The following primers were used to generate seven partial clones: Fragment 1: XLTBP1 U1; 5′-GGC GGC CGC ACC ATG GAT ACC AAG CTC ATA-3′; xLTBP1-D1; 5′-GGC ACT GTG GTA AGA GAA CT-3′, Fragment 2: xLTBP1-U2; 5′-TGG GAG TTC CTC GTC TAC AC-3′, xLTBP1-D2 5′-CAG GAA CAG GGC ATT TGT CG-3′, Fragment 3: xLTBP-U3: 5′-TCT GCT GTT GCA CCG TGG GA-3′; xLTBP1-D3: 5′-TTT TCA CAT CGG CCA TGG GA-3′, Fragment 4: xLTBP1-D4: 5′-ATT GAT CCC CAG ATG GAG CT-3′, xLTBP1-U4: 5′-AAT GAA TGC GCC AAG GAT CC-3′, Fragment 5: xLTBP1-D5a: 5′-CTG AAG TAG TAA GAC CTT GT-3′, xLTBP1-U5a: 5′-CCT AAT GGG GAA TGT CTC AA-3′, Fragment 6: xLTBP1-U6: 5′-GAG CTG GAT GGG GAG ATA AT-3′, xLTBP1-D6: 5′-GTC TAT AAG AGC ATC ATG TC-3′, Fragment 7: xLTBP1-U7: 5′-GAC TAT GCC GAA CTT TGC AA-3′, xLTBP1-D7: 5′-GGC GGC CGC TAT TAC TCA AGT TCA CTG TCG-3′. The fragments were assembled by using unique restriction sites to yield the full-length clone. Primers for RT-PCR analysis were: xLTBP 3s; 5′-CCT ATG ACA GGG TAT TGC CGA-3′ and xLTBP1-D5, 5′-ATT CAT CAG AAC CTA TGA C-3′. These primers amplify both the long and short forms of Xenopus LTBP-1, 286 and 208 base pairs, respectively. RT-PCR assays were as described previously (Altmann *et al.*, 2001; Wilson and Hemmati-Brivanlou, 1995). Primers: chordin (Chd) (Sasai *et al.*, 1994), Wnt8 (Smith and Harland, 1991), OtxA (otx) (Lai *et al.*, 1995), Brachyury (xBra) (Smith *et al.*, 1991), Xhox3 (Ruiz i Altaba and Melton, 1989), goosecoid (gsc) (Cho *et al.*, 1991), Sox17a (Hudson *et al.*, 1997), ornithine decarboxylase (ODC) forward 5′-CAA CGT GTG ATG GGC TGG AT-3′, reverse 5′-CAT AAT AAA GGG TTG GTC TCT GA-3′. The sequence of xLTBP has been submitted to Gen Bank.

In Situ Hybridization

Antisense RNA probe was prepared by linearizing xLTBP-1 CS2++ with *Sal*I and transcribing with T3 RNA polymerase according to manufacturer's instructions with digoxygenin. Whole-mount *in situ* hybridization was performed as described (Harland, 1991). Embryos to be sectioned were embedded in 20% gelatin/PBS and cut on a vibratome at either 50 or 100 μm.

RNA Preparation, Injection, and Microsurgery

RNA was prepared *in vitro* by using Ambion Message Machine and the appropriate polymerase from the following vectors: xLTBP1 CS2++ *Asc*I/Sp6, hLTBP1 (Kanzaki *et al.*, 1990), pSP64T activin-HA EcoRI/Sp6, (Liem, Jr. *et al.*, 1997), xNRI CS2++ *Not*I/Sp6. Embryos were injected at the stage indicated in the figure legends, and tissue explants were isolated and processed for RT-PCR as described previously (Kay and Peng, 1991). To prepare soluble proteins, Xenopus ovaries were surgically removed from anesthetized adult females and oocytes were manually dissected. Oocytes were injected with 50 pg of capped mRNA, cultured overnight in OR2+ media containing 100 μg/ml acetylated BSA. Tissue explants were dissected from injected or uninjected embryos from the regions indicated in the figure legends and cultured to the stages indicated. Explants and embryos were cultured in 0.1–0.5× MMR (Peng, 1991)

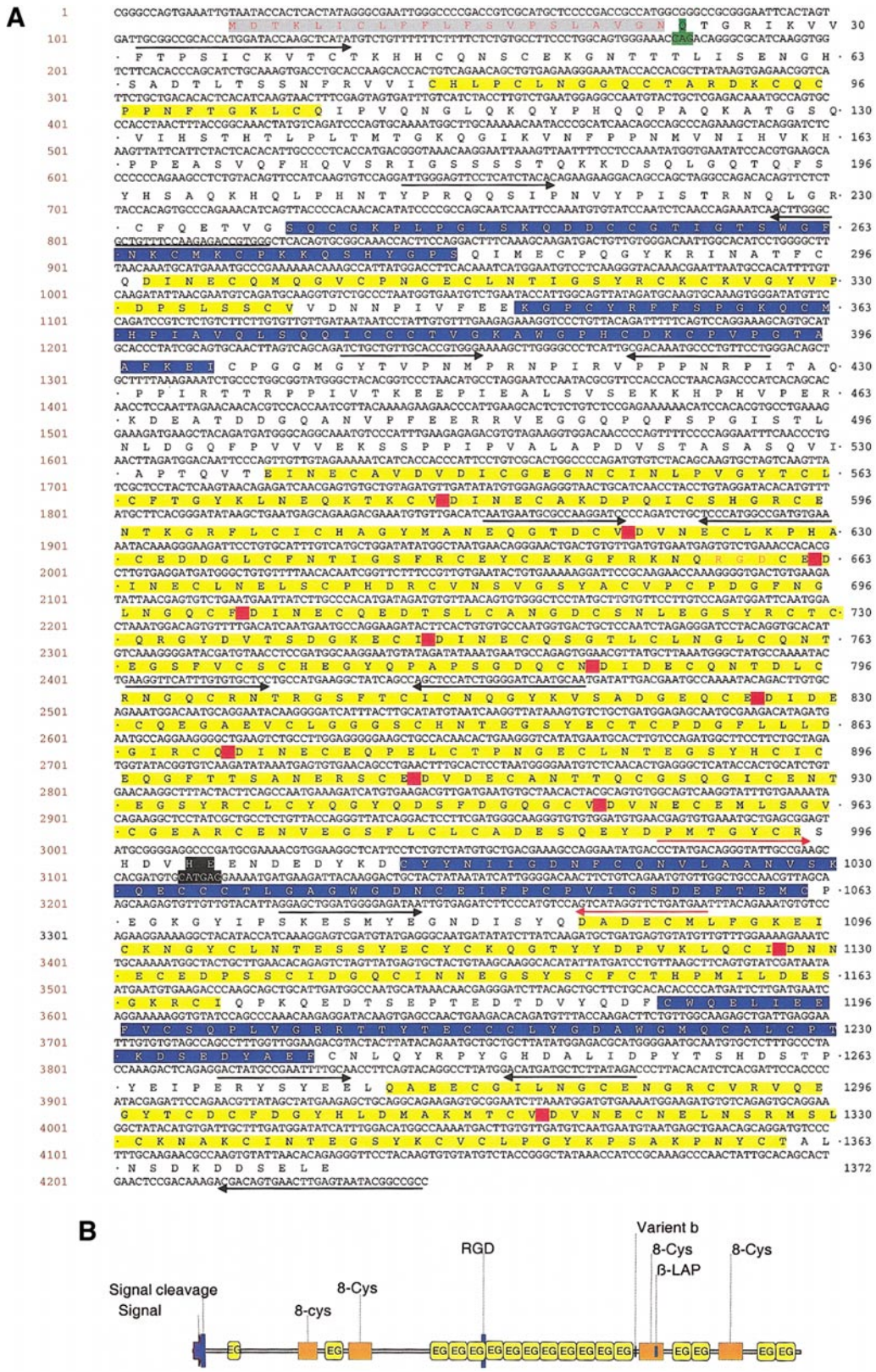
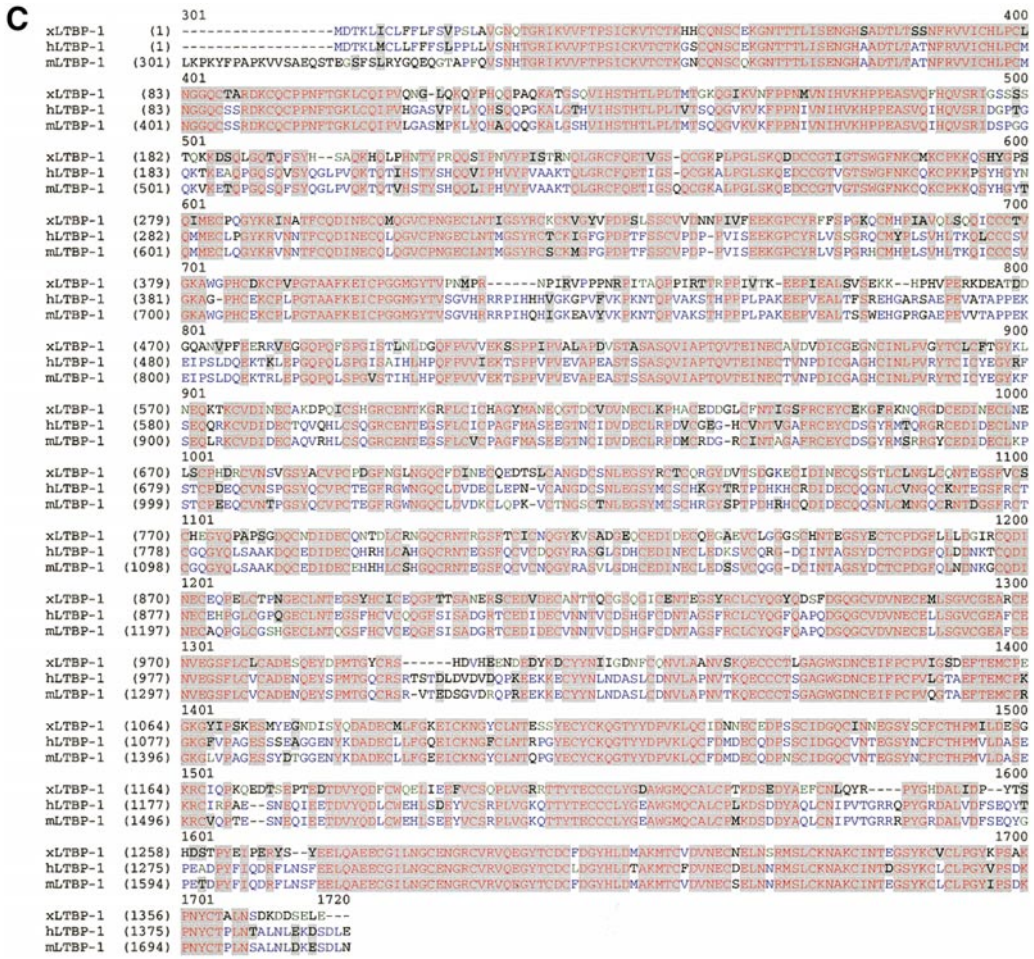


FIG. 1. *Xenopus* LTBP-1 sequence and analysis. (A) Primary sequence of the short form of Xenopus LTBP-1 and its translation product. Primers used to isolate the cDNA are indicated by arrows. Red arrows indicate the RT-PCR primers. Color key: yellow boxes indicate



EGF-like domains predicted by SMART with the domains separated by red boxes; red text indicates RGD cell binding site; gray box is the signal sequence; green box is the signal cleavage site; blue boxes are cysteine-rich repeats; the black box indicates the location of the 25-amino-acid deletion GTNSFGESNNDSLLNTFSPSVTHDNT. (B) Diagram of *Xenopus* LTBP-1 indicating the domain structure. (C) Sequence alignment of *Xenopus*, human, and mouse LTBP-1. Conserved residues are boxed. Blue text indicates amino acids conserved between two of the three species. Bold text indicates amino acid similarity.

Cell Culture and Conditioned Media

Serum-free conditioned media from CHO cells or CHO cells expressing human LTBP-1 (clone 22 gift of J. Annes) were concentrated 20-fold in a Centricon-100 (Amicon). Concentrated media were diluted 2-fold in 0.5× MMR (to promote healing of the dissected animal caps) and applied to animal cap explants dissected at the blastula stage (stage 9) (Kay and Peng, 1991). Recombinant activin protein (produced in oocytes) was added at a dose sufficient to induce ventral mesoderm marker expression. Explants were incubated for 2 h at room temperature, and then placed at 16°C overnight, prior to harvesting the RNA at stage 13.

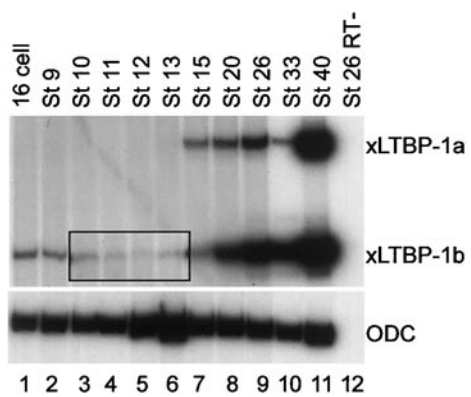


FIG. 2. RT-PCR analysis of *Xenopus* LTBP-1 expression. Temporal expression of *Xenopus* LTBP-1. The two alternative forms are detected by using a single primer pair indicated in Fig. 1A. Transcripts are detected after 25 cycles. ODC is a loading control.

RESULTS

Cloning and Characterization of xLTBP-1 cDNAs

To isolate the xLTBP-1, a cDNA library derived from the *Xenopus* XTC cell line was screened by using a human

LTBP-1 cDNA as a probe. A number of positive clones derived from this screen encoded sequences homologous to the human LTBP-1. Specific primers derived from the XTC clones were generated to cover the complete cDNA and assemble the open reading frame (ORF) of xLTBP-1 into the expression vector pCS2++ (see Materials and Methods). The nucleotide and amino acid sequences of xLTBP-1 are shown in Fig. 1A. xLTBP-1 contains 1418 amino acids and has, in addition to the signal sequence, 4 cysteine rich (CR1, -2, -3, -4) domains and 17 EGF-like repeats. This domain structure (Fig. 1B) is homologous to the human, mouse, and rat forms of LTBP-1 (Koli *et al.*, 2001; Saharinen *et al.*, 1999). Interestingly, a second xLTBP-1 clone derived from the Xenopus cell line has an insertion amino-terminal to the third CR3 domain of 26 amino acids with the sequence ^{101}GTNSFGESNNDSLLNTFSPSVTHDNT^{127}. This variant of LTBP-1 has not been described in any other species. The alignment of amino acid sequences between human, mouse, and Xenopus (when the extra sequences in the CR3 domain are excluded) shows that the proteins share about 65% identity (Fig. 1C).

Embryonic Expression of xLTBP-1

To address the temporal expression pattern of the two xLTBP-1 RNAs during embryonic development, we used RT-PCR analysis to screen embryos of different stages. A single primer pair designed to detect both sets of transcripts (with and without the insertion before the third CR3 domain) was used to determine whether there was a difference in the timing of expression of the two RNAs. Figure 2 shows that the short form of xLTBP-1 (xLTBP-1b) is expressed maternally (lane 1) as early as the 16-cell embryo stage, prior to the initiation of zygotic transcription (lane 2). Expression of the RNA continued throughout zygotic development. Interestingly, between stages 10 and 15, expression decreased but increased in later stages. The long form of xLTBP-1 (xLTBP-1a) was not expressed maternally, but begins to be expressed during gastrulation at the same time as the short form. Therefore, expression of the two alternatively spliced forms of xLTBP-1 is temporally regulated.

We next asked whether xLTBP-1 mRNA is expressed in a spatially restricted manner during embryogenesis. In order to address this question, we performed *in situ* hybridizations on whole embryos using an antisense xLTBP-1 probe that recognizes both transcripts. We found that xLTBP-1 RNA expression is dynamic during embryogenesis but is never ubiquitous. Figure 3A shows that xLTBP-1 message is detected at the start of gastrulation in the dorsal mesoderm in the organizer. Within the organizer, expression is confined to the cells of the axial mesoderm, which later form the prechordal plate and notochord (Fig. 3B; see *). Following gastrulation, during neural plate stages, the expression of xLTBP-1 RNA is restricted to the posterior half of the embryo (Fig. 3C). At this stage, no expression is seen in the anterior neural plate. At stage 25, transcripts are detected within the spinal cord and but not in the forebrain (Fig. 3D). A section through the spinal cord shows strong expression in the axial mesoderm and the neural tube (Fig. 3E). By stage 28, xLTBP-1 is detected within the forebrain, hindbrain, and spinal cord (Fig. 3F). A posterior to anterior gradient of expression is observed in the somites of the paraxial mesoderm. At this stage, xLTBP-1 is expressed in both the roofplate (see white arrow, Fig. 3G) as well as the floorplate (black arrow) of the posterior region of the spinal cord. In addition to the axial mesodermal expression, xLTBP-1 transcripts begin to be expressed in the anterior wall of the telencephalon, roof plate of the midbrain, and rhombomeres 1, 3, and 5 (Fig. 3H). By stage 35, cranial expression of xLTBP-1 RNA also includes pharyngeal pouches 1, 2, and 3, the photoreceptors of the eye, the cement gland, cranial ganglia, and nerves VII (Geniculate) and IX (Glossopharyngeal) (Fig. 3I). Finally, at tailbud stages, a high level of RNA is expressed in the tailbud or cordoneural hinge, a region previously demonstrated to be endowed with organizing activity (Fig. 3J). Expression can also be detected in the heart (see white arrow).

To examine the spatial expression of the two xLTBP-1 forms during gastrula stages by a biochemical approach, we isolated the dorsal and ventral marginal zones of a stage 11 gastrula and performed RT-PCR analysis using the same primers as for Fig. 2A but for additional cycles to enhance the signal. The results from this experiment confirmed xLTBP-1 expression at gastrula stage 11 (Figs. 2 and 3K). Expression was shown to be primarily restricted to the dorsal marginal zone and only low levels of expression were observed in either the animal pole or ventral marginal zone. In addition, expression of the long form is restricted to the dorsal marginal zone. The additional markers included in Fig. 3K are controls for the dissection and rule out contamination. Thus, the two forms of xLTBP-1 mRNA show both temporal and spatial expression specificity. The common denominator among the cells expressing xLTBP-1 RNA is that they belong to organizing centers in the embryo. Given the ubiquity of various TGF-β superfamily members throughout the embryo, the highly dynamic but restricted expression of xLTBP-1 supports the hypothesis that xLTBP-1 activities are limited to specific TGF-β family ligands.

xLTBP-1 Synergizes with Activin and Xnr1 in Mesoderm Induction

To characterize the embryological activity of LTBP-1, we tested the inducing ability of both human and *Xenopus* LTBP-1 in the context of ectodermal explants. When microdissected from blastula-stage embryos, animal cap explants will differentiate into epidermis (Wilson and Hemmati-Brivanlou, 1995). However, if these explants are exposed to activins or nodals, they adopt a mesodermal fate (Gurdon *et al.*, 1994). To test whether LTBP-1 affected cell fate, we first performed a dose-response experiment in which increasing concentrations of sense RNA encoding hLTBP-1 were microinjected in the animal poles of two-cell-stage blas-

tomeres (Figs. 4A and 4B). The injected embryos, along with their sibling controls, were allowed to develop to blastula stages, at which point animal caps were removed, cultured until controls reached gastrula stages, and assayed for the expression of cell type molecular markers by RT-PCR. When injected alone, even at high doses (2 ng/embryo), hLTBP-1 mRNA does not induce molecular markers for endoderm (Sox 17α), organizer (cerberus, goosecoid, or chordin), mesoderm (Xbra), and ventral mesoderm (Xwnt8 and Xhox3) (Fig. 4A, lane 2; Fig. 4B lane 3). This result suggests that this hLTBP-1 RNA cannot independently induce mesodermal or endodermal cell fates in pluripotent explants. In contrast, activin RNA, when injected alone at low concentrations, induces the expression of ventral mesoderm and endoderm markers (Fig. 4A, lane 3; Xwnt8 and Xhox3, Sox17α). As the concentration of activin is increased, markers of dorsal mesoderm and organizer are detected in addition to the ventral markers (Fig. 4A, lanes 4 and 5, cerberus, goosecoid, chordin). When activin and hLTBP-1 are coexpressed, the level of activin required to induce the expression of dorsal markers is reduced to the lowest amount tested (0.5 pg activin). Similar enhancement is observed when LTBP is coexpressed with AXnr-1 in which the prepro region of activin is fused to Xenopus nodal related-1 (Fig. 4A, lanes 9–14). To determine whether the observed interaction was due to the presence of the activin prepro region, we examined the effect of hLTBP-1 on native Xenopus Xnr-1 (Fig. 4B). When injected alone, Xnr-1 did not induce the organizer genes chordin and cerberus nor the endoderm marker Sox17α (Fig. 4B, lane 1). When Xnr-1 and were hLTBP-1 coinjected, high levels of expression of chordin, cerberus, and Sox17α were observed. In addition, expression of the ventral marker Xwnt8 was decreased, while the pan-mesodermal marker Brachyury was induced. These results demonstrate synergism between hLTBP and both activin and nodal during mesoderm induction.

In addition to increasing the relative activity of activin, the coexpression of activin and hLTBP-1 changes the character of the mesoderm induced. Whereas high concentrations of activin result in the expression of dorsal markers together with ventral markers (Fig. 4A, lane 5), coexpression with hLBTP-1 suppresses the expression of the ventral markers (Fig. 4A, lane 8; Wnt8 and Xhox3), while enhancing the expression of dorsal markers (Fig. 4A, lane 8; cerberus, goosecoid, chordin). These changes in the character of the induced mesoderm are also observed when Xnr-1 is coexpressed with hLTBP-1 (Fig. 4B, lanes 1 and 2). This effect can take place by increasing the overall concentration of these molecules, therefore promoting more dorsal fates, both by affecting the secretion of these ligands, as well as through direct extracellular interactions (as we have demonstrated for LTBP-1 and activin). However, whether the extracellular binding of LTBP-1 to activin changes the properties of activin signaling is unknown, although plausible. For instance, this could have an effect at the level of differential receptor interaction, competitive binding for activin antagonists, etc. Similarly, it is unclear whether LTBP-1 can bind multiple TGF-β ligands simultaneously, and what effects this might have in mesodermal patterning. Most organizer-specific secreted factors are inhibitors of signaling pathways. For example, follistatin inhibits activin and BMP11 signaling (Fainsod *et al.*, 1997; Hemmati-Brivanlou *et al.*, 1994), DKK inhibits the Wnt pathway (Glinka *et al.*, 1998), Cerberus inhibits BMP, nodal, and Wnt signaling (De Robertis *et al.*, 1997; Glinka *et al.*, 1997), and Noggin, Chordin, and Xnr3 inhibit BMP/GDF signaling (Wessely and De Robertis, 2002). Therefore, we tested whether LTBP-1 was acting as an inhibitor using the animal cap assay. We found that LTBP-1 does not act as a neural inducer when expressed in ectodermal cells (data not shown). This eliminated the possibility that LTBP-1 was acting as an inhibitor of the BMPs, since inhibition of BMP/GDF signaling is the signature of neural induction in amphibian explants. Thus, in addition to synergism between activin/nodal, the thresholds for induction of mesoderm are differentially affected by the presence of LTBP.

To test whether LTBP-1 can interact with activin in the extracellular space, in addition to its role in secretion of TGF-β ligands, we prepared both activin and hLTBP-1 proteins and assayed them in the context of the animal cap explants. When activin protein is added alone to tissue explants at low doses, ventral mesoderm is induced (Fig. 5, lane 5; xBra, Wnt8, Hox-B9). hLTBP-1 protein from conditioned medium mixed with activin protein induces the expression of dorsal markers (Fig. 5 lane 6; chordin, myoD, coll-type II). Thus, LTBP protein acts as an agonist modifier of activin ligands. This result demonstrates that LTBP-1 can interact with ligands in the extracellular space in a manner independent of its interactions in the secretory pathway.

DISCUSSION

Xenopus LTBP-1 is expressed in two temporally and spatially regulated forms during development. The short form is expressed maternally and levels of this transcript decline through blastula stages until zygotic expression of both the long and short forms is detected. The localization of xLTBP-1 to the dorsal region, and in particular the dorsal marginal zone, suggested the involvement of xLTBP-1 in mesoderm and neural induction. In animal cap explants, the RNA injection of our LTBP-1 RNA was not capable of inducing either mesoderm or neural tissues, suggesting that the presence of the appropriate ligand was required. Indeed, we have shown that xLTBP-1 enhances the activities of activin and Xnr1.

In Vivo Function of LTBP-1 in Embryonic Axis Formation

A possible *in vivo* function of xLTPB1 is to potentiate locally the activities of the organizer specific mesoderm inducers of the activin and nodal pathways. The ability of

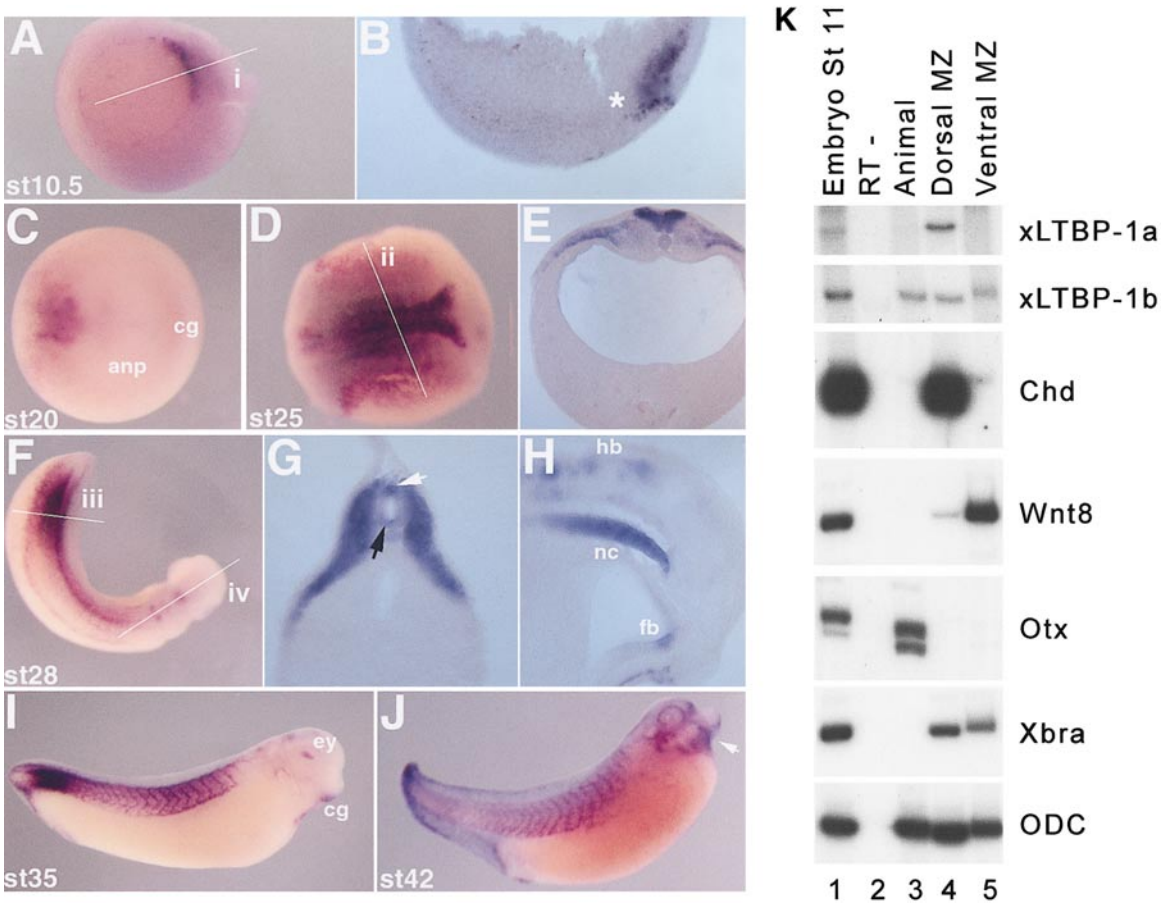


FIG. 3. *In situ* hybridization of *Xenopus* LTBP-1. (A–J) *In situ* hybridization. (A): Stage 10.5 (gastrula). Expression of xLTBP-1 is detected within the organizer region. (B) A section (depicted by the line "i" in A) shows expression is restricted to the dorsal mesoderm of the organizer (*). (C) Stage 20. Expression of xLTBP-1 in the spinal cord. No expression is seen in the anterior neural plate at this stage. Anterior/ventral is to the right. (D) Stage 25. Transcripts can be detected throughout the spinal cord region but still not in anterior domains. (E) A section through the embryo at the level of the spinal cord (indicated by the line "ii" in D); xLTBP-1 RNA expression is seen throughout the neural tube but not in the notochord at this stage. (F) Expression is also detected in the lateral plate mesoderm, Stage 28. Expression is seen in the tail, throughout the spinal cord and in the hindbrain. (G) Transverse section through the spinal cord ("iii" in F); xLTBP-1 RNA is expressed in the mesoderm lateral to the neural tube, in the notochord and in both the roof (white arrow) and floor plates (black arrow). (H) Midsagital section through the head (line "iv" in F). xLTBP-1 RNA is also expressed in anterior regions, in the hindbrain, and in the ventral forebrain. Note the expression within the notochord. (I) Stage 35. xLTBP-1 RNA is highly expressed in the muscle blocks surrounding the spinal cord. It is also expressed in the rhombomeres of the hindbrain, the eye, and the cement gland. High expression is present in the tailbud cordoneural hinge. (J) Stage 42. Additional xLTBP-1 RNA expression is observed in more anterior regions, including the heart (white arrow), the branchial arches, and around the eye. anp, anterior neural plate; cg, cement gland; ey, eye; fb, forebrain; hb, hindbrain; he, heart; nc, notochord. Locations of the sections are indicated by dashed lines. (K) xLTBP-1 RNA expression in dissected gastrula embryos: Dorsal and ventral marginal zones and animal cap explants were dissected from stage 11 gastrula embryos and analyzed by RT-PCR. xLTBP-1 was amplified by using the same primers as in Fig. 2 but for 30 cycles. The following controls are included as markers for the dissected fragments: Chordin (chd) for dorsal mesoderm, Wnt8 for ventral mesoderm, Otx for animal cap, Brachyury (xBra) as a pan mesodermal marker, and ODC as the loading control.

LTBPs to interact with the extracellular matrix (Koli *et al.*, 2001; Saharinen *et al.*, 1999; Unsold *et al.*, 2001) suggests that xLTBP-1 may restrict the diffusion of activin/nodal molecules in both the embryo and the formation of the primary axis, resulting in higher local levels of activity of these two signaling molecules. A requirement of LTBP for the formation of the axis is not directly addressed in these studies. However, the coordinate expression of xLTBP-1 with its cognate ligands suggests that xLTBP-1 may be required for proper activin/nodal function in the developing embryo. This hypothesis is consistent with the embryonic lethal phenotype observed with the loss-of-function of

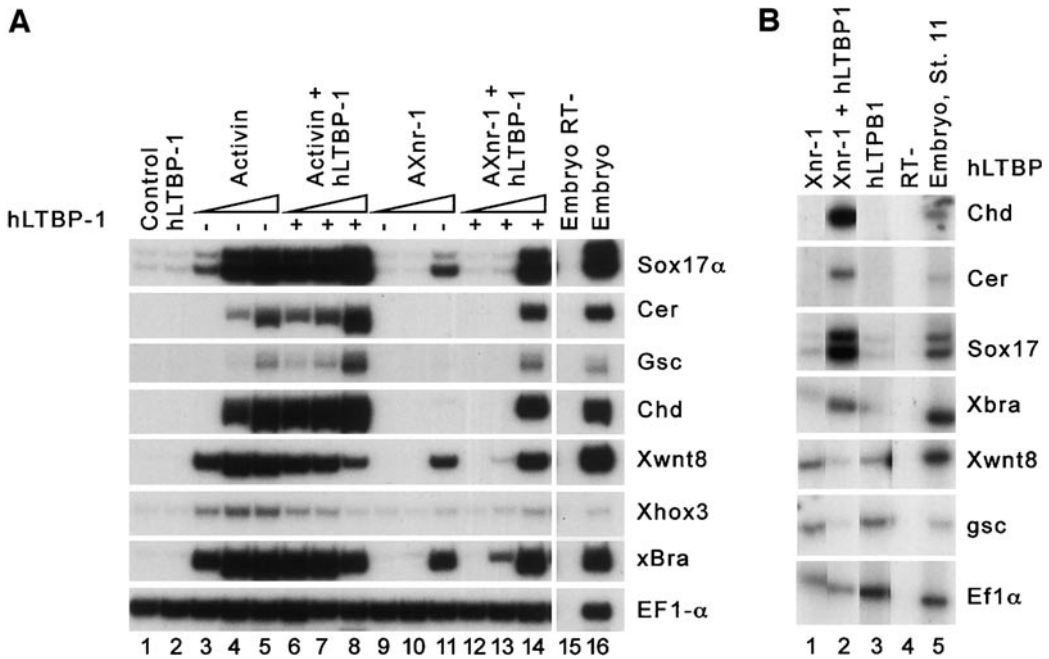


FIG. 4. Gain-of-function analysis of hLTBP-1 in animal cap explants. (A) Coexpression of hLTBP-1 with TGF-β family ligands: Embryos were injected in the animal pole of both blastomeres at the two cell stage. Amounts of RNA injected were: 0.2 ng hLTBP-1; 0.5, 1.0, and 2.0 pg activin with and without hLTBP-1; 0.1, 0.2, 0.5 ng AXnr-1 with and without hLTBP-1. Animal explants were isolated at stage 9 and cultured until sibling controls reached stage 11 and processed for RT-PCR as described. (B) Coexpression of hLTBP-1 with Xenopus nodal related-1. Xnr-1 (100 pg/embryo) was injected with or without hLTBP-1 (500 pg/embryo). Animal explants were isolated at stage 9 and cultured until sibling controls reached stage 11 and processed for RT-PCR as described. xBra, brachyury (pan-mesodermal marker); Cerberus, Chordin (dorsal mesendodermal markers); goosecoid (dorsal mesoderm marker); XWnt-8 (ventral mesodermal marker); Xhox3 (ventral mesodermal marker); Sox17α (endodermal marker); EF1-α, elongation factor alpha (loading control).

LTBP-2 (Shipley *et al.*, 2000). Further studies are required to examine the ligand specificities of xLTBPs. However, the differences in activities observed for TGF-β ligands and their receptors strongly support the idea of additional ligand binding in the LTBP family.

Role of LTBP-1 in Signaling among the Cells of the Organizer

Our studies also demonstrate the localization of the long form of xLTBP-1 to the organizer and illustrate the ability of xLTBP-1 to act as an agonist in both activin and nodal signaling. Thus, in the context of the organizer, LTBP-1 would be required for organizer function. Interestingly, antagonists of TGF-β signaling are also expressed at the same time and place as the ligands and their binding proteins. The consequences of expression of these competing activities is not known but may contribute to maintaining a high local ligand concentration and activity, while inhibiting activin/nodal signaling at a distance.

LTBP-1 in the TGF-β Signaling Cascade

The LTBPs are important for the secretion of TGF-β1, -2, and -3 and this requires covalent interactions between the two molecules (Dabovic *et al.*, 2002; Flaumenhaft *et al.*, 1989; Miyazono *et al.*, 1991). No noncovalent interactions between the TGF-βs and LTBPs have been reported and attempts to show such interactions were inconclusive (unpublished observations). Our finding that conditioned medium containing LTBP-1 can potentiate the activity of activin indicates a physical interaction between activin and LTBP that enhances the potency of activin signaling. Although we have not yet demonstrated direct physical association between LTBP-1 and activin, the data suggest that the LTBPs and the related family members, the fibrillins, may have a role in controlling the activities of additional TGF-β superfamily members. The recent report (Arteaga-Solis *et al.*, 2001) describing syndactyly related to a failure of BMP-4 signaling in mice containing a null mutation in the fibrillin-2 gene is consistent with such an interaction.

As noted above, the organizer is a source of antagonists (follistatin), the signaling molecules (activin/nodal), and agonists (LTBP-1). It is not clear how these three kinds of molecules interact within the organizer. We are currently examining whether LTBP/follistatin/activin ternary complexes form and interaction of LTBP with activin blocks follistatin binding to activin.

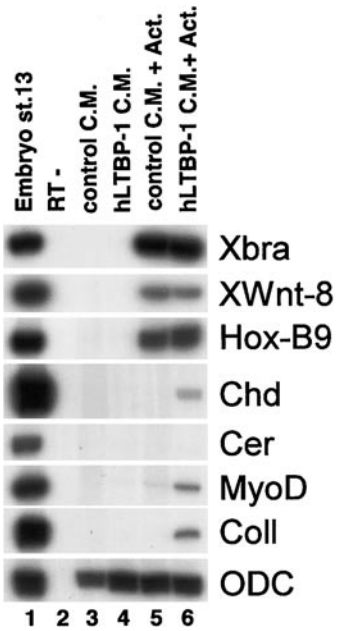


FIG. 5. Soluble hLTBP-1 can promote the effect of recombinant activin in animal cap explants. Dissected blastula-stage animal caps were exposed to conditioned media from hLTBP-1-producing CHO cells (lane 4) or from CHO cells alone (lane 3), in the absence (lanes 3 and 4) or presence of recombinant activin protein made in oocytes (lanes 5 and 6). The concentration of activin protein applied to the caps in this experiment promotes ventrolateral mesodermal fates only (lane 5). In the presence of hLTBP-1-conditioned medium, dorsal markers are expressed in the caps, indicating that hLTBP-1 and activin can functionally cooperate in solution (lane 6). xBra, brachyury (pan-mesodermal marker); XWnt-8 (ventral mesodermal marker); Hox-B9 (lateral mesodermal marker); Chordin, Cerberus (dorsal mesendodermal markers); MyoD (muscle marker); Collagen-type II (notochord marker); ODC, ornithine decarboxylase (loading control).

Morphogen Thresholds

Recent studies have demonstrated that both activin and nodals can act as morphogens establishing different fates at different activity thresholds (Chen and Schier, 2001; Kodjabachian, 2001; McDowell *et al.,* 2001). We have shown that LTBP-1 modulates the morphogen activities of activin and Xnr-1, and potentially other related ligands. Therefore, LTBPs might function to promote the morphogenic activities of these molecules in the embryo. This is important in the context of activin signaling, as the role of activin as a morphogen has been questioned because of its expression pattern in the mesoderm. Whereas nodals are confined to the organizer region, activins are expressed ubiquitously in the embryo as illustrated by *in situ* hybridization. Morphogen experiments have demonstrated that high thresholds of activin give rise to axial dorsal mesoderm, whereas low levels yield mesoderm of ventral character (Gurdon *et al.,* 1994). Our results indicate that the higher threshold of activin activity might be created by LTBP in the organizer. If we assume that activin protein distribution is homogenous in the embryo (i.e., reflects the distribution of the RNA as detected by *in situ* hybridization), the presence of LTBP-1 in the dorsal side aspect will specifically increase activin activity in the organizer cells. It is thus tempting to speculate that the two extremes of the activin gradient are modulated dorsally by processing factors such as LTBP and ventrally by the BMP/GDFs, which are expressed in the ventral lateral region of the embryo. The fact that our study shows that LTBP-1 can specifically interact with activin is in support of LTBP being involved in the high threshold gradient of activin activity. Future studies will address how LTBP activity helps refine activin/nodal morphogen signaling in the context of other modulators of TGF-β signaling in the embryo.

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